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Recombinant expression of soluble murine prion protein for C-terminal modification

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ABSTRACT

Membrane attachment of prion protein (PrP) via its glycosylphosphatidylinositol (GPI) anchor plays a key role during conversion of cellular PrP^{C} into its pathogenic isoform PrP^{Sc}. Strategies to access homogenous lipidated PrP via expressed protein ligation (EPL) are required to fully decipher the effect of membrane attachment. Such strategies suffer from insoluble expression of PrP-intein fusion constructs and low folding efficiencies that severely limit the available amount of homogeneous lipidated PrP. Here, we describe an alternative method for expression of soluble PrP-intein fusion proteins in *Escherichia coli* that provides access to natively folded PrP ready to use in EPL.

1. Introduction

Prion diseases or transmissible spongiform encephalopathies (TSEs) are infectious neurodegenerative disorders (e.g. scrapie in sheep, BSE in cattle and CJD in humans), which are caused by conversion of cellular, predominantly α-helical prion protein (PrP^{C}) into a toxic, β-sheet-rich isoform (PrP^{Sc}) [1–5]. Typically, PrP^{C} can be found anchored to the outer leaflet of cell membranes via its C-terminally attached glycosylphosphatidylinositol (GPI) anchor. The biological function of PrP^{C} itself and its attachment to cellular membranes is not yet fully understood. However, membrane attachment of PrP^{C}, especially the preference for ordered membrane domains, plays an important role in converting PrP^{C} to PrP^{Sc} and strategies to generate homogeneous membrane-anchored PrP variants by expressed protein ligation have been developed [6,7]. This finding is supported by the fact that both PrP^{C} and PrP^{Sc} were found in ordered membrane domains extracted from either infected cells or infected mouse brain [8–11]. Furthermore, Baron et al. proved that these ordered membrane domains (rafts) act as a "meeting place" between PrP^{C} and PrP^{Sc}. Conversion only occurs when both protein variants are inserted into contiguous membranes [2]. Recently, Goold et al. developed a new cell-based system in which epitope-tagged PrP^{C} is expressed in a wt-PrP-knockdown neuronal cell line. This cell line was exposed to RML prion and it was found that the formation of de novo epitope-tagged PrP^{Sc} primarily occurred on the plasma membrane within ordered membrane domains [3].

Even though there is conclusive evidence demonstrating the influence of membrane attachment of PrP via its GPI anchor on the conversion process available, most in vitro studies still use recombinant PrP without a membrane anchor [12–17] or heterogeneous posttranslationally modified PrP^{C} extracted from neuronal cells [2,18]. In order to generate membrane attached PrP variants, we have previously developed semisynthetic strategies to create homogeneous lipidated PrP^{C} by combining recombinant protein technology and chemically synthesized GPI mimics [6,7,19]. These strategies rely on expression of PrP in fusion with an *Mxe* intein in *Escherichia coli* in order to generate PrP with a C-terminal α-thioester group, which is required for expressed protein ligation (EPL) with membrane anchors, or on the use of the DnaE split intein. However, these constructs are typically deposited in inclusion bodies in *E. coli*, probably due to the structurally important disulfide bridge in PrP [20]. Generation of PrP α-thioesters require solubilizing these inclusion bodies under denaturing conditions, which severely limits the yield of the intein cleavage and subsequent EPL reactions.

In order to improve semisynthetic access to posttranslationally modified PrP, we have set out to develop soluble expression of

Abbreviations: ASP, 6xHis-ATPase-SrtA-PrP; ASPM, 6xHis-ATPase-SrtA-PrP-*Mxe*Intein-6xHis-CBD; CBD, chitin binding domain; GPI, glycosylphosphatidylinositol; GSH, reduced glutathione; GSSG, oxidized glutathione; HDP, 6xHis-DnaK-TEV-PrP; IPTG, isopropyl-β-D-thiogalactopyranoside; MESNA, sodium 2-mercaptoethanesulfonate; NaOAc, sodium acetate; NBD, 4-chloro-7-nitrobenzofurazan; PMSF, phenylmethylsulfonyl fluoride; RML, Rocky Mountain Laboratories; SBD, substrate binding domain; SrtA, Sortase A

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PrP-*Mxe* intein constructs in *E. coli*. Access to larger amounts of soluble, folded PrP will allow more straightforward access to homogeneously modified PrP variants, e.g. carrying a C-terminal membrane anchor and/or N-glycosylations. Efforts to achieve this goal by co-expression of PrP with bacterial chaperones in the cytoplasm of *E. coli* in order to assist the folding of prion protein failed [21].

However, previous experiments by others have shown that a direct N-terminal fusion of *E. coli* DnaK chaperone leads to an improved solubility of recombinantly expressed proteins in *E. coli* [21]. Therefore we cloned, expressed and tested a variety of N-terminal fusion constructs with PrP-*Mxe* intein constructs.

2. Materials and methods

2.1. Materials

All chemicals used were purchased from Sigma–Aldrich, Roche and Merck-Chemicals in the highest purity available if not otherwise stated. The Sortase A expression plasmid pET30b-SrtA is a kind gift from Professor Teruyuki Nagamune [22].

2.2. Plasmid construction

Plasmid pTXB3-PrP containing a mouse PrP(90–231)-encoding gene [6] was used as template for cloning work. The *E. coli* K12 DnaK-encoding gene was N-terminally fused to a gene coding for PrP(90–231) to give plasmid pET30-HDP. The DnaK-substrate binding domain was removed from pET30-HDP and a TEV cleavage site replaced with a SrtA-site in pET30-ASP plasmid. This plasmid was extended by a C-terminal fusion to give an intein fusion construct in plasmid pET30-ASPM (for details see Supplementary material).

2.3. Protein expression and purification

All protein constructs are expressed in 2xYT medium after IPTG induction at 25 °C overnight (for details see Supplementary material). Purification of all constructs was carried out as described for 6xHis-DnaK (Supplementary material).

2.4. Protease cleavage reactions

Purified HDP protein was cleaved by TEV protease as previously described [23]. Here we used a ratio of HDP and TEV of 30:1 (w:w) in 50 mM Tris–HCl, pH 8.0, 0.5 mM EDTA, 2 mM PMSF and 3 mM/0.3 mM GSH/GSSG, and incubated at 10 °C overnight. Sortase A cleavages were carried out in 50 mM Tris–HCl, pH 8.0 buffer containing 5 mM $CaCl_2$, 10 mM Gly–Gly–Gly (Sigma) and 2 mM PMSF at room temperature, overnight (Supplementary material).

2.5. Circular dichroism (CD) spectroscopy

Far-UV CD spectra were recorded at 22 °C on an Applied Photophysics Chirascan Plus system. PrP proteins were diluted in 20 mM NaOAc at pH 5.5 to a final concentration of 12 μM. CD spectra were collected at a scan speed of 20 nm/min at 1 nm bandwidth and response time of 4 s. All spectra were recorded in a 0.1 cm cuvette between 195 and 260 nm. All data shown here represent the average of 10 recorded spectra.

2.6. Preparation of ASP containing a C-terminal α-thioester

Purified ASPM protein was immobilized on chitin beads and cleaved by addition of 250 mM sodium 2-mercaptoethanesulfonate (MESNA) in 50 mM Tris–HCl, pH 8.0 at 4 °C overnight. The resulting ASP containing C-terminal α-thioester (ASP-SR) found in the flow through of the cleavage reaction was submitted to a Superdex 75 (16/60) column equilibrated with 50 mM Tris–HCl buffer at a flow rate of 0.5 ml/min. Pure fractions of ASP-SR were pooled and concentrated by using ultrafiltration (Amicon 30KDa MWCO, Millipore).

2.7. Expressed protein ligation

Expressed protein ligation (EPL) was carried out in 50 mM Tris–HCl, pH 8.0, 2% (v/v) ethanethiol with concentrations of 50 μM ASP-SR and 500 μM NBD peptide at room temperature for 24 h. Reactions were analyzed by SDS–PAGE followed by fluorescence scanning and coomassie staining. Reaction mixtures were dialyzed against 50 mM Tris–HCl, pH 8.0 buffer and Sortase A was added.

3. Results and discussion

3.1. Construction of DnaK-PrP fusion protein, expression and purification

The plasmid expressing *E. coli* DnaK with an N-terminal 6xHis tag (Fig. 1) was constructed as described above. The resulting plasmid (pET30-DnaK) is transcribed under control of an IPTG inducible T7 promoter. Expression and purification of this His-DnaK construct provided 14 mg of His-DnaK from 1 l medium in 3 h after induction (Fig. S1A). Subsequently, the mouse PrP(90–231) encoding gene was cloned into pET30-DnaK to form plasmid pET30-HDP. Expression of the resulting His-DnaK-PrP (HDP) construct was evaluated by SDS–PAGE. IPTG-induced overexpression of HDP provides the target protein mainly in the soluble fraction (Fig. 2A) which is in good agreement with a similar strategy previously applied for soluble expression of full-length PrP [21]. HDP protein was purified by Ni–NTA affinity chromatography followed by size exclusion chromatography (SEC) using a Superdex 200 (16/60) column to achieve high purity in combination with an excellent overall yield of 42 mg HDP from 1 l of *E. coli* culture (overnight expression, Fig. 2B).

3.2. Cleavage of DnaK from DnaK-PrP

Purified HDP protein was cleaved by addition of recombinantly expressed TEV protease (see Supplementary material). Favorable redox conditions for this cleavage reaction as well as for maintaining the crucial internal disulfide bridge in PrP were achieved by addition of the redox couple GSH/GSSG. Protease cleavage was monitored by SDS–PAGE and was complete after overnight incubation at 10 °C. Even though control of the redox conditions helped to stabilize PrP, a subsequent centrifugation step was required to remove precipitated PrP. Approximately 10% of PrP precipitated under these conditions. Separation of the remaining soluble PrP from the DnaK-tag and from TEV protease was expected to work via Ni–NTA affinity purification. PrP should be collected in the flow-through while DnaK and TEV should bind to the Ni–NTA matrix via their respective His-tags. However, this strategy for separating PrP from DnaK was unsuccessful in our hands. Only minor amounts of soluble PrP were found in the flow-through and the majority of PrP eluted together with DnaK at concentration of 250 mM imidazole (Fig. 2C). This behavior can be explained by a rather strong interaction between the substrate binding domain (SBD) of DnaK with PrP as observed in other cases [24,25]. Based on this assumption we removed the DnaK-SBD and speculated that the ATPase domain by itself would impart similar solubilizing properties as the complete DnaK protein.

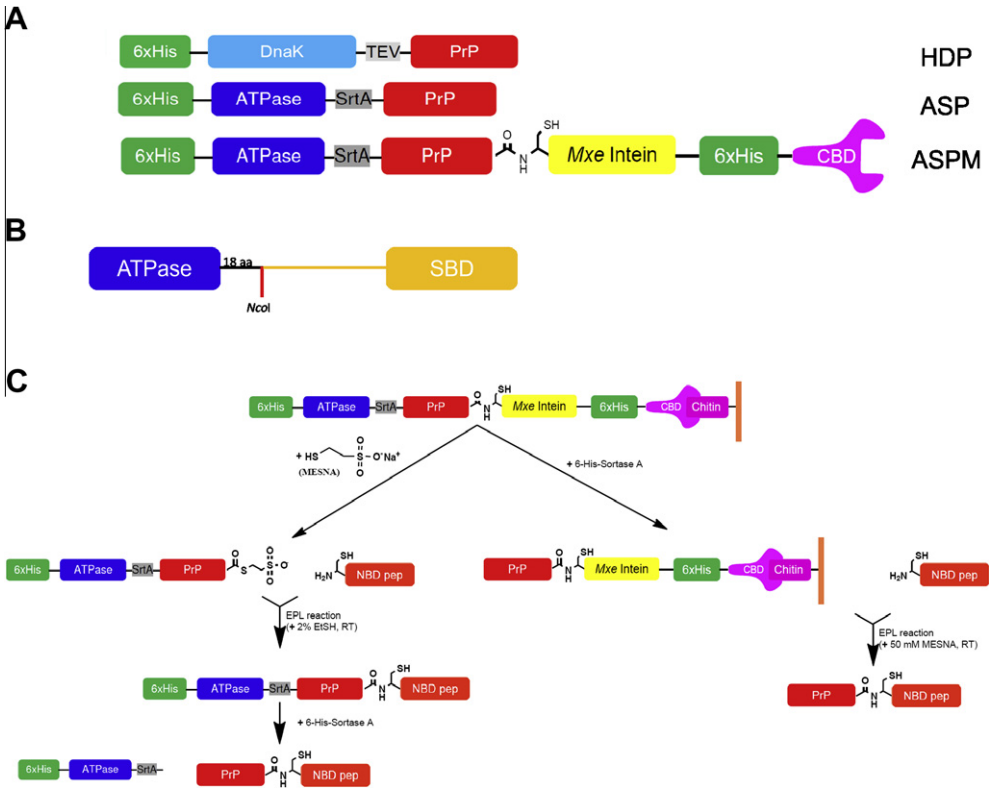

Fig. 1. (A) Scheme of all generated fusion proteins. (B) *Nco*I site in DnaK. (C) Semisynthesis strategy to create soluble PrP with C-terminal modifications.

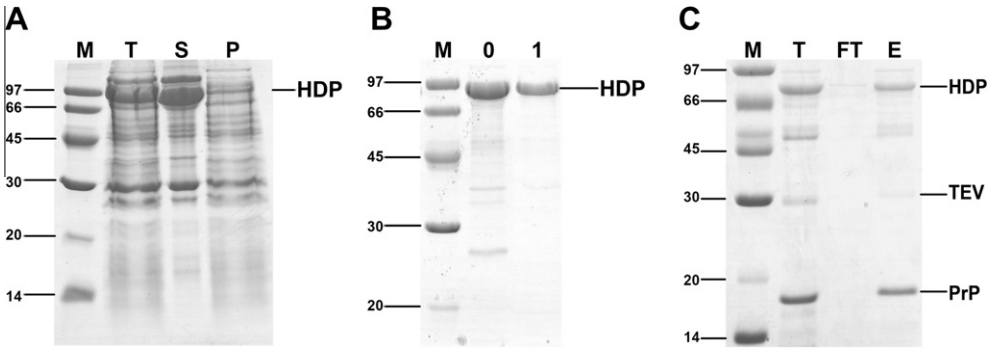

Fig. 2. SDS–PAGE analysis of soluble DnaK-PrP fusion protein (HDP). (A) Expression, M: mfolecular weight marker; T: total cell extract, S: soluble fraction, P: pellet fraction. (B) Purification of HDP, 0: Ni–NTA eluate, 1: pure fraction from SEC. (C) TEV cleavage, T: total reaction mix, FT: flow through, E: elution after incubation with Ni–NTA resin.

3.3. Removal of SBD from DnaK-PrP

To this end we could take advantage of a natural *NcoI* site ideally positioned just at the beginning of the SDB within the DnaK gene (Fig. 1B). Moreover, we replaced the TEV protease cleavage site with a Sortase A (SrtA) site to circumvent the use of reducing agents during TEV cleavage reactions, which causes partial precipitation of PrP as described above. These changes led to the ATPase-SrtA-PrP (ASP) construct, which was tested for soluble expression in two *E. coli* strains: BL21(DE3)RIL and Origami 2 (DE3) RIL. The latter one has been described as an *E. coli* strain that greatly enhances disulfide bridge formation in the cytoplasm [26]. Here, we did not recognize any significant differences in solubility and expression levels between both strains (Fig. 3A). Based on this comparison we conclude that the ATPase domain of DnaK in the ASP construct is sufficient to ensure soluble overexpression in the cytoplasm of *E. coli*.

Purification was now achieved by Ni–NTA affinity chromatography as described above and provided highly pure ASP protein with an average yield of 85 mg/l culture (Fig. 3B). This finding confirms our initial assumption that the SBD of DnaK mediates tight binding to PrP but plays only a minor role in solubilizing it. Cleavage of purified ASP protein by SrtA was optimized by screening several conditions (Fig. S2A). Optimized conditions resulted in quantitative release of soluble PrP from the ASP construct (Fig. S2B). The identity of PrP was confirmed by western blot analysis (Fig. S1B).

3.4. High yield production of soluble PrP(90–231) from ASP construct

Optimal conditions for expression, cleavage and purification of soluble PrP as identified above were applied for processing 85 mg of pure ASP obtained from 1 l of *E. coli* culture to produce soluble PrP. Only minor amounts of PrP are retained on the Ni–NTA matrix via unspecific interaction (Fig. 3C). Removal of the SBD almost completely alleviates the problem of PrP not eluting from to the Ni–NTA matrix.

Next, the secondary structure of soluble PrP was analyzed by CD spectroscopy and compared to recombinant PrP that was refolded

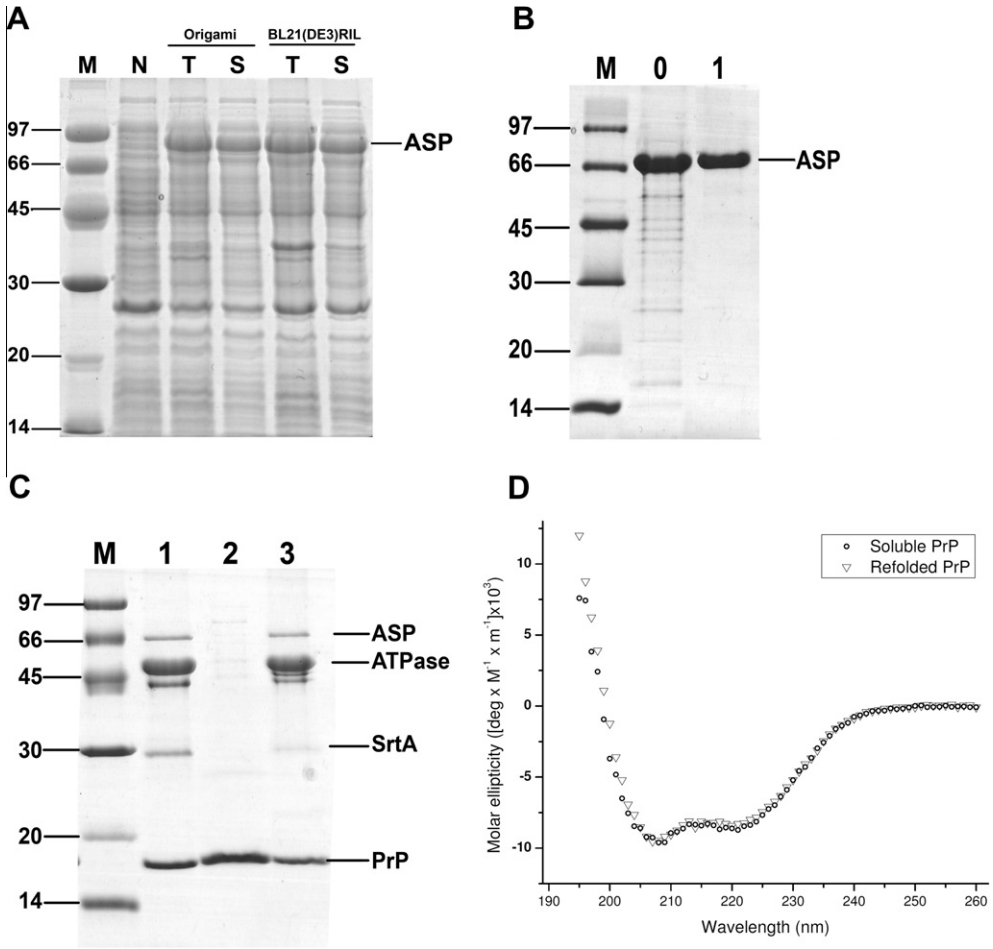

Fig. 3. SDS–PAGE analysis of ATPase-PrP fusion protein (ASP). (A) Soluble expression in two *E. coli* strains: BL21(DE3)RIL and Origami2(DE3)RIL, N: control without IPTG, T: total cell extract, S: soluble fraction of lysed cells. (B) Purification of ASP, 0: Ni–NTA eluate, 1: pure fraction from SEC. (C) SrtA cleavage, 1: total reaction mix, 2: flow through, and 3: elution from Ni–NTA resin. (D) CD spectra of soluble PrP from ASP construct (circles) and refolded PrP protein (triangles) from inclusion bodies.

from inclusion bodies following an established procedure. Both variants showed similar CD spectra with 2 minima at 208 and 222 nm indicating predominantly α-helical proteins (Fig. 3D). Quantitative analysis indicates a similar percentage of α-helical structure elements in both PrP variants, typical for cellular PrP^C protein [27,28]. The overall yield of soluble PrP protein from this strategy was 15 mg from 1 l of *E. coli* culture (69% based on the amount of total PrP expressed), indicating the superior results obtained with this strategy when compared to another recently published soluble expression system for PrP [29]. Furthermore, an aggregation experiment, based on Thioflavin T binding of fibrils, for soluble, refolded and denatured PrP variants was conducted to compare their biochemical characteristics. Soluble and refolded PrP exhibit a similar fibrillation behavior with an extended lag phase between 30 and 40 h, very different from that of denatured PrP with a lag-phase of ca. 10 h (Fig. S4). In combination with the secondary structure analysis this experiment emphasizes the similar properties of soluble and refolded PrP.

3.5. Preparation of soluble PrP containing a C-terminal α-thioester

The successful strategy for expression of large amounts of soluble PrP(90–231) was extended to soluble expression of a PrP-intein construct. To this end we added a C-terminal *Mxe* mini-intein-CBD tag to ASP. Soluble expression of this ASPM construct in *E. coli* BL21(DE3)RIL was confirmed by analysis of centrifugation fractions of lysed cells. ASPM accumulated mostly in the supernatant (Fig. 4A). Ni–NTA purification yielded ASPM that was subsequently immobilized on chitin beads via its C-terminal CBD (Fig. 4B). PrP expression was also confirmed by western blot analysis (Fig. S1B). Addition of 250 mM MESNA led to cleavage of the *Mxe* mini-intein and to spontaneous formation of an α-thioester group at the C-terminus of the ASP protein. The resulting ASP-SR was found in the supernatant (Fig. 4C) and further purified by SEC using a Superdex 75 (16/60) column to reach high purity (Fig. 4D). The presence of an α-thioester group at the C-terminus of ASP was confirmed by successful expressed protein ligation (EPL) with a fluorescently labeled peptide (NBD-peptide, analytical data in Fig. S3) containing an N-terminal cysteine residue (Fig. 1C) [30]. SDS–PAGE analysis in combination with a fluorescence scan of the gel prior to coomassie staining clearly proved that the ASP protein is almost quantitatively linked to the NBD peptide (Fig. 4E). In a final step, the EPL reaction mix was incubated with SrtA in order to release soluble PrP carrying the NBD peptide. After incubation for 16 h the reaction mix was centrifuged and the supernatant was again analyzed by SDS–PAGE (Fig. 4F). Almost quantitative ligation and subsequent complete removal of the ATPase domain make this strategy much more efficient than previously used systems based on refolded PrP and even split inteins [6].

To study the efficiency of the above described reaction in reverse order (Fig. 1C), 4 mg of ASPM protein were immobilized on 2 ml of chitin beads and the ATPase domain was cleaved off by SrtA to produce PrP-intein on chitin beads (Fig. 5A). Immobilized PrP-intein was mixed with 100 μM NBD peptide (Fig. S3) and 50 mM MESNA in buffer containing 50 mM Tris–HCl at pH 8.0, 1 mM EDTA and 0.5 mM TCEP. The in situ cleavage and ligation reaction was

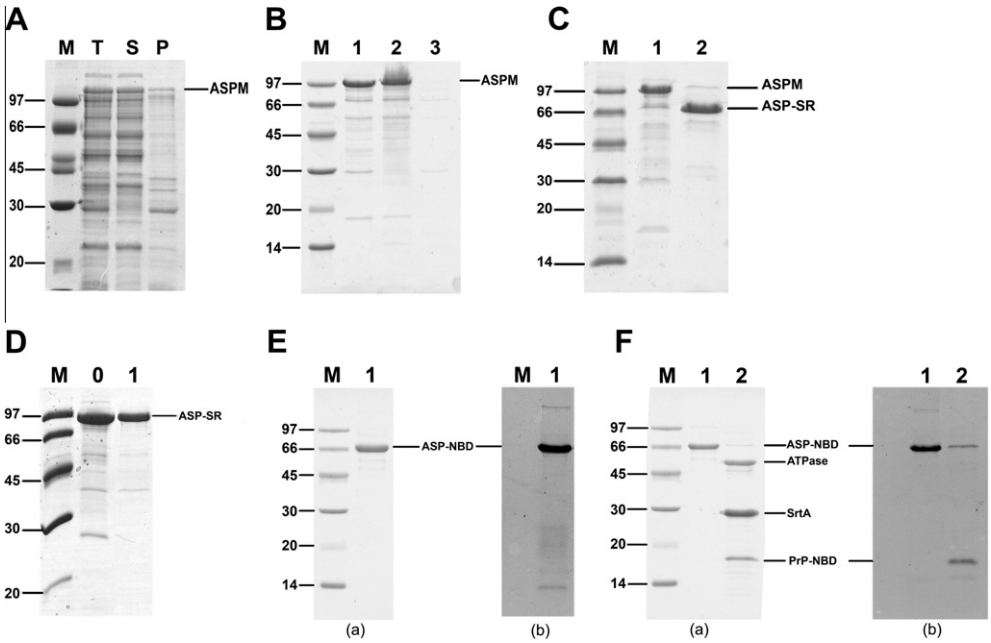

Fig. 4. SDS–PAGE analysis of ASPM and ASP-SR in EPL reactions. (A) ASPM expression, T: total cell extract, S: soluble fraction, P: pellet fraction of lysed cells. (B) 1: Ni–NTA purifications of ASPM, 2: ASPM immobilized on chitin beads, 3: flow through. (C) MESNA-induced cleavage of ASPM, 1: ASPM protein, 2: ASP-SR released from chitin beads. (D) ASP-SR purification by SEC, 0: crude ASP-SR, 1: pure fraction. (E) EPL reaction of ASP-SR with NBD peptide; (a) coomassie staining, (b) fluorescence scan. (F) ASP-NBD after SrtA cleavage; (a) coomassie staining and (b) fluorescence scan.

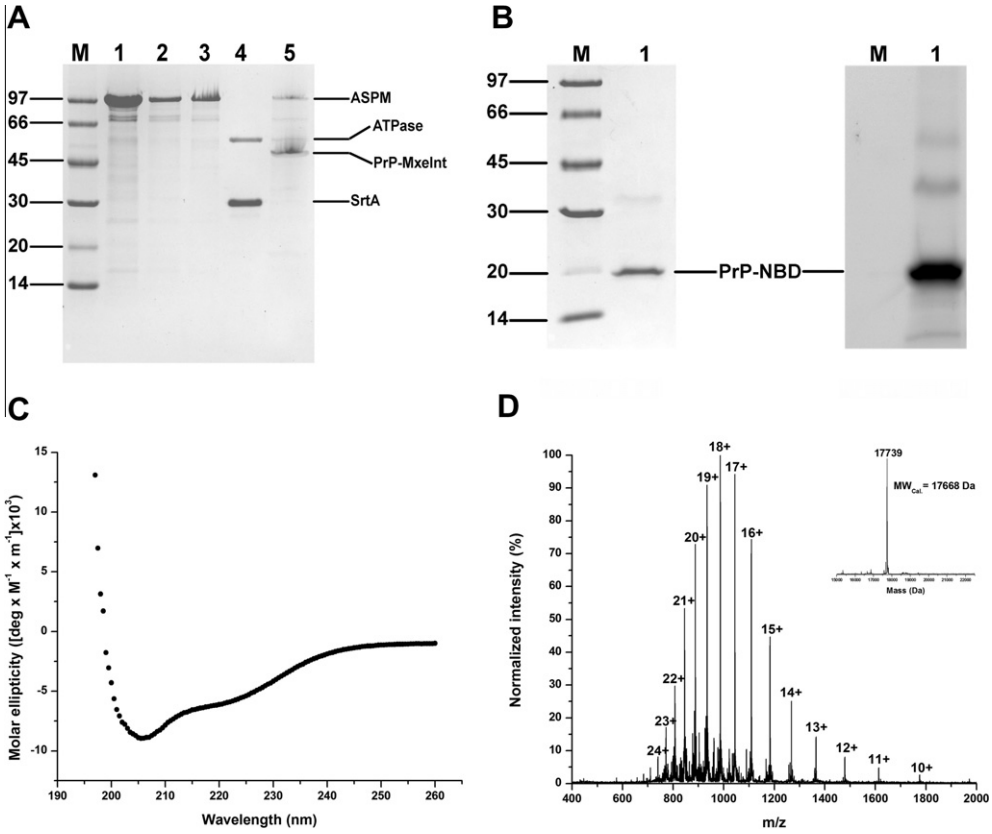

Fig. 5. C-terminal modification of soluble PrP. (A) SDS–PAGE of SrtA cleavage of immobilized ASPM protein. 1: ASPM used for loading chitin beats, 2: excess ASPM not bound to beads, 3: ASPM immobilized on chitin beads, 4: SrtA addition leads to release of ATPase domain, 5: PrP-intein bound to chitin beads. (B) SDS–PAGE analysis of soluble PrP-NBD generated by in situ cleavage and EPL reaction with NBD peptide and subsequent purification by Ni–NTA chromatography. Coomassie staining (a) and fluorescence scan (b); (C) CD spectrum of PrP-NBD; (D) ESI-MS of soluble PrP-NBD.

carried out over 24 h with gentle shaking. The solution containing the ligation product PrP-NBD was centrifuged to remove chitin beads, dialyzed against buffer (50 mM Tris–HCl, pH 8.0) to remove excess NBD peptide and loaded on Ni–NTA resin to obtain purified

PrP-NBD (Fig. 5B). Soluble PrP carrying a C-terminal NBD peptide was obtained with an overall yield of 250 μg from 4 mg of ASPM protein (32% based on the total amount of PrP that can theoretically be obtained from ASPM protein). Next, the secondary structure of soluble PrP-NBD was analyzed by CD spectroscopy, which showed the typical α-helical features of correctly folded cellular PrP (Fig. 5C). ESI-MS analysis of PrP-NBD protein gave a molar mass of 17 739 Da (Fig. 5D), which deviates by +71 Da from the calculated mass of 17 668 Da. Such a difference was observed only when TCEP was added to the in situ cleavage and ligation reaction over extended periods of time at a concentration of 0.5 mM to prevent oxidation of the N-terminal cysteine residue of the NBD peptide. TCEP has long been recognized as a well-tolerated and widely used reducing agent that does not cause significant side reactions under many conditions [31]. However, here at pH 8.0 TCEP or an undetectable by product causes a defined modification of the PrP, which does not occur in the absence of TCEP.

4. Conclusion

We developed the soluble expression of a recombinant PrP-*Mxe* intein fusion protein (ASPM) to improve the efficiency of semisynthetic strategies for producing homogenously posttranslationally modified PrP. We showed that PrP-*Mxe* intein protein N-terminally fused to the ATPase domain of HSP 70 DnaK overexpresses as a soluble protein in the cytoplasm of *E. coli*. Consequently, this fusion allows a straightforward way to generate PrP-variants containing C-terminal modifications without the need for subsequent protein folding and disulfide bond formation. The latter step can be especially challenging when introducing additional cysteine residues, e.g. during EPL reactions that increase the probability of non-native disulfide bond formation. We further improved this strategy of soluble expression by minimizing the DnaK fusion partner down to the ATPase domain alone. This leads to high quantities of soluble, cellular PrP even in the presence of a C-terminal intein fusion as well as preventing undesired strong interactions between the DnaK-SBD and PrP. Previous studies reported that neither co-expression with chaperones nor periplasmic expression in *E. coli* succeeded in producing soluble intact PrP proteins [21,32]. Thus, we believe that this strategy can be extensively applied for soluble expression of other proteins containing disulfide bridges in fusion with intein for semisynthesis method.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.febslet.2012.12.026.

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